

## CsrA and Cra Influence *Shigella flexneri* Pathogenesis<sup>∇</sup>

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***Shigella flexneri* is a facultative intracellular pathogen that invades and disrupts the colonic epithelium. In order to thrive in the host, *S. flexneri* must adapt to environmental conditions in the gut and within the eukaryotic cytosol, including variability in the available carbon sources and other nutrients. We examined the roles of the carbon consumption regulators CsrA and Cra in a cell culture model of *S. flexneri* virulence. CsrA is an activator of glycolysis and a repressor of gluconeogenesis, and a *csrA* mutant had decreased attachment and invasion of cultured cells. Conversely, Cra represses glycolysis and activates gluconeogenesis, and the *cra* mutant had an increase in both attachment and invasion compared to the wild-type strain. Both mutants were defective in plaque formation. The importance of the glycolytic pathway in invasion and plaque formation was confirmed by testing the effect of a mutation in the glycolysis gene *pfkA*. The *pfkA* mutant was noninvasive and had cell surface alterations as indicated by decreased sensitivity to SDS and an altered lipopolysaccharide profile. The loss of invasion by the *csrA* and *pfkA* mutants was due to decreased expression of the *S. flexneri* virulence factor regulators *virF* and *virB*, resulting in decreased production of *Shigella* invasion plasmid antigens (Ipa). These data indicate that regulation of carbon metabolism and expression of the glycolysis gene *pfkA* are critical for synthesis of the virulence gene regulators VirF and VirB, and both the glycolytic and gluconeogenic pathways influence steps in *S. flexneri* invasion and plaque formation.**

The Gram-negative bacterium *Shigella flexneri* is a causative agent of shigellosis, a severe infection of the colonic epithelium. *S. flexneri* is primarily transmitted between hosts via the fecal-oral route to its infective site in the colon. Following attachment to colonic epithelial cells, *S. flexneri* induces its own uptake and replicates in the host cell cytosol. The bacterium then spreads directly to adjacent epithelial cells, thereby propagating itself within the intestinal epithelium (24). *S. flexneri* invasion requires genes on the 220-kb virulence plasmid (50, 51). These include the Ipa effectors necessary for bacterial entry into colonic epithelial cells (32) and the type III secretion system that delivers the effectors to the host cytosol (3, 33, 34, 65).

The ability of *S. flexneri* to sense and respond to environmental changes is an important aspect of its pathogenesis. During intracellular growth, *S. flexneri* modulates expression of over one-quarter of its genes (30), including genes required for carbon source uptake and utilization (30, 45). The gene encoding the hexose-phosphate transporter, *uhpT*, was significantly upregulated in *S. flexneri* growing inside the eukaryotic cell (30, 45). This transport system should allow intracellular shigellae to take up glucose-phosphate for glycolysis, yet the expression of glycolytic genes was downregulated in the bacteria during intracellular growth (30). This may indicate that *S. flexneri* consumes both glycolytic and nonglycolytic carbon sources during intracellular growth, perhaps at different stages

of an infection. Little is known about *Shigella* carbon metabolism in the colon, a site at which the bacterial cells are adapting to the host environment and initiating expression of genes required for invasion of the epithelium. Carbon metabolism regulators may coordinate expression of central carbon metabolism with external nutrient availability and bacterial metabolic demands. Regulators of central carbon metabolism have been studied in detail in *Escherichia coli* K-12, and this pathway was used as the basis for analysis of the close relative *S. flexneri*.

The *E. coli* carbon storage regulator CsrA is a global regulator of cellular metabolism (Fig. 1A) (43). CsrA acts posttranscriptionally to increase glycolysis while repressing both gluconeogenesis and glycogen metabolism (46). CsrA is a 6.8-kDa RNA-binding protein that functions as a homodimer to modulate mRNA stability (4, 11, 63). The mechanism by which CsrA inhibits the glycogen biosynthesis gene *glgC* has been studied in detail. CsrA binds two sites in the *glgC* mRNA 5' leader sequence, one of which overlaps the Shine-Dalgarno sequence, interfering with ribosome loading and translation initiation (35) and enhancing decay of the message (4). CsrA acts as a positive regulator of glycolysis, and disruption of *csrA* led to decreased activity of the glycolytic enzymes glucose-6-phosphate isomerase, triose-phosphate isomerase, and enolase (46), although the mechanism by which CsrA positively regulates the activity of these enzymes has not been fully determined (66).

The noncoding RNAs CsrB and CsrC regulate the activity of CsrA by sequestering the protein and preventing its binding to target mRNAs (54, 67) (Fig. 1A). *E. coli* CsrB contains 22 copies of the predicted CsrA-binding site, whereas CsrC contains 9 copies (67). Expression of *csrB* and *csrC* is activated by the BarA-UvrY two-component system, which is regulated by CsrA. In response to signals, including the sugar metabolism end products formate and acetate (9), the histidine kinase

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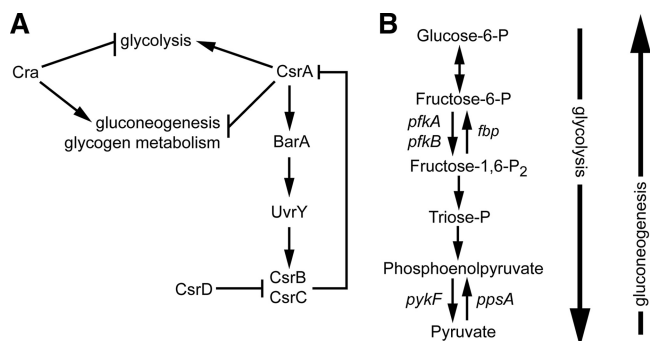


FIG. 1. *E. coli* central carbon metabolism is regulated by CsrA and Cra. (A) The transcriptional regulator Cra inhibits expression of glycolytic genes while activating expression of genes involved in gluconeogenesis. CsrA regulates stability of mRNAs for genes involved in glycolysis and gluconeogenesis. The noncoding RNAs CsrB and CsrC sequester the CsrA protein from its targets. Expression of *csrB* and *csrC* is induced by the BarA-UvrY two-component system, which is activated by CsrA. CsrD decreases the stability of both CsrB and CsrC. Based on their genetic relatedness and the data gathered in this study, CsrA and Cra likely regulate metabolism similarly in *S. flexneri* and *E. coli*. (Illustration modified from reference 2 with permission of the publisher). (B) Irreversible steps in the metabolic pathways allow for independent regulation of glycolysis and gluconeogenesis. The opposing effects of CsrA and Cra are due in part to their reciprocal regulation of the glycolytic gene *pfkA* and the gluconeogenic gene *ppsA*.

BarA phosphorylates its cognate response regulator UvrY, which activates transcription of both *csrB* and *csrC* (39, 53). The levels of CsrB and CsrC are regulated by CsrD protein, which promotes RNaseE-dependent turnover of CsrB and CsrC (25, 53). By stimulating production of BarA, leading to increased levels of its antagonists, CsrA autoregulates its activity via a larger feedback pathway.

An additional carbon metabolism regulator, Cra, inhibits expression of genes involved in glycolysis while activating expression of gluconeogenic genes (47). Thus, the effect of Cra on carbon metabolism is opposite that of CsrA (Fig. 1A). Cra is a transcriptional regulator that binds to palindromic sequences in the operators of genes in its regulon. The effect of Cra on gene expression is influenced by the position of the Cra operator sequence relative to the RNA polymerase-binding site. If Cra binds upstream of the RNA polymerase-binding site, Cra promotes transcription of the downstream operon. However, if the Cra-binding site overlaps or is downstream of the polymerase-binding site, Cra inhibits transcription (47). Additionally, glycolytic metabolites affect Cra activity, as binding of the glycolytic metabolites fructose-1-phosphate or fructose-1,6-bisphosphate to Cra prevent it from binding its DNA targets (42).

One of the primary control points of CsrA and Cra regulation is phosphofructokinase (Pfk). Although several reversible enzymes function in both glycolysis and gluconeogenesis, the reaction catalyzed by the glycolytic enzyme Pfk is irreversible under physiological conditions (Fig. 1B). Pfk catalyzes the phosphorylation of fructose-1-phosphate to fructose-1,6-bisphosphate. In *E. coli*, the major enzyme, PfkI, encoded by the *pfkA* gene, accounts for 90% of total cellular phosphofructokinase activity (19). CsrA and Cra control the glycolytic and gluconeogenic pathways by regulating the level of PfkI and a

second enzyme, Pps, which converts pyruvate to phosphoenolpyruvate (46, 48) (Fig. 1B).

Optimal growth and survival of *S. flexneri* in its host likely require both efficient utilization of available carbon sources and rapid response to changing carbon sources. This work examines the role of CsrA and Cra in *S. flexneri* pathogenesis in a cell culture model and demonstrates that expression of the glycolysis gene *pfkA* is required for synthesis of *S. flexneri* virulence proteins and invasion of cultured cells. The effect on virulence occurs at the level of VirF and VirB, the master regulators of *S. flexneri* virulence gene expression.

MATERIALS AND METHODS

**Media and growth conditions.** Strains and plasmids used in this study are shown in Table 1. All strains were routinely maintained at -80°C in tryptic soy broth (TSB) supplemented with 20% (vol/vol) glycerol. *E. coli* strains were grown in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 1% NaCl) or on LB agar plates. *S. flexneri* was grown on TSB agar containing 0.01% (wt/vol) Congo red (CR) to select CR<sup>+</sup> colonies. For assays comparing the wild-type and mutant strains, isolated colonies from Congo red agar were inoculated into LB broth and grown at 37°C with shaking to exponential phase (*A*<sub>650</sub>, 0.75 to 0.80). The growth rates were monitored by measuring the *A*<sub>650</sub>, and under these conditions, the growth rates for the wild-type and mutant strains were the same. Antibiotics were added at the following concentrations per milliliter: 25 µg ampicillin, 30 µg chloramphenicol, 1.6 µg tetracycline, and 50 µg kanamycin. To examine glycogen accumulation, bacteria were grown on Kornberg medium agar (1.1% K<sub>2</sub>HPO<sub>4</sub>, 0.85% KH<sub>2</sub>PO<sub>4</sub>, 0.6% yeast extract) containing 1% (wt/vol) glucose. Plates were then inverted over iodine crystals to stain accumulated glycogen with iodine vapor (44).

Henle cells (intestinal 407; American Type Culture Collection) were cultured

TABLE 1. Strains and plasmids

Strain or plasmid	Description	Source and/or reference
<i>E. coli</i> strains		
JW0078	<i>cra</i> ::Kan	Keio collection (1)
JW1899	<i>uvrY</i> ::Kan	Keio collection
JW2757	<i>barA</i> ::Kan	Keio collection
JW3221	<i>csrD</i> ::Kan	Keio collection
JW3887	<i>pfkA</i> ::Kan	Keio collection
TRMG1655	<i>csrA</i> ::Kan	44
RG1-B	<i>csrB</i> ::Cm	15
TWGM1655	<i>csrC</i> ::Tet	68
<i>S. flexneri</i> 2a strains		
2457T	Wild type	Walter Reed Army Institute of Research
AGS100	2457T <i>barA</i> ::Kan	This study
AGS110	2457T <i>uvrY</i> ::Kan	This study
AGS120	2457T <i>csrA</i> ::Kan	This study
AGS130	2457T <i>csrB</i> ::Cm	This study
AGS140	2457T <i>csrC</i> ::Tet	This study
AGS150	2457T <i>csrD</i> ::Kan	This study
AGS190	2457T <i>cra</i> ::Kan	This study
AGS220	2457T <i>pfkA</i> ::Kan	This study
Plasmids		
pGEM-T Easy	PCR cloning vector	Promega
pQE2	Cloning vector; IPTG-inducible expression from T5 promoter	Qiagen
pWKS30	Low-copy-number cloning vector	63
pWSK29	Low-copy-number cloning vector	63
pQCSrB	2457T <i>csrB</i> in pQE2	This study
pQCSrC	2457T <i>csrC</i> in pQE2	This study
pQCSrA	2457T <i>csrA</i> in pQE2	This study
pWCsrA	2457T <i>csrA</i> in pWSK29	This study
pWCra	2457T <i>cra</i> in pWKS30	This study
pWPfkA	2457T <i>pfkA</i> in pWSK29	This study

TABLE 2. Primers used in this study

Primer	Sequence
csrA-fwd-BseRI	5'-ATCTGAGGACGACAAAGTATGCTGATTCTG-3'
csrA-rev-HindIII	5'-AAGCTTGGGTGCGTCTCACCATAAAG-3'
pQCsrB-fwd	5'-GAATTCATCTTCGTCGACAGG-3'
pQCsrB-rev	5'-AAGCTTAAAAGTCCGCGAAGGATAGC-3'
pQCsrC-fwd	5'-GAATTCCAAAGCGTAAAGTAGCACCC-3'
pQCsrC-rev	5'-AAGCTTCTTTGCATGACCTTTGCTGCG-3'
pWPfkA-fwd	5'-GTGGACAGGGAGGGTAAACGGTCTATG-3'
pWPfkA-rev	5'-GCGGCCCGCTGCGGGTATATGTTGAGGG-3'
pWCra-fwd	5'-GAATTCGCAAAATCAGTGGGAAC-3'
pWCra-rev	5'-GCGGCCCGCTTGGCAGCATTACCTTG-3'
For real-time PCR	
virB2-for	5'-TCCAATCGCGTCAGAACTTAACT-3'
virB2-rev	5'-CCTTTAATATTGGTAGTGTAGAATAAGAGATTC-3'
virB2 probe	FAM-5'-AGGACTTGAAAAGGC-3'-MGBNFQ
VirF-fwd	5'-TCGATAGCTTCTTCTTCTAGTTTCTG-3'
VirF-rev	5'-GAAAGACGCCATCTCTCTCGAT-3'
VirF probe	FAM-5'-TCAGATAAGGAAGATTGTTGAAA-3'-MGBNFQ

in minimal essential medium (MEM; Gibco) supplemented with 10% Bacto tryptone phosphate broth (Difco, Becton Dickinson and Company, Franklin Lakes, NJ), 10% fetal bovine serum (Gibco), 2 mM glutamine, and 1× nonessential amino acids (Gibco). Henle cells were incubated at 37°C with 95% air–5% CO<sub>2</sub>.

**Bacterial strains and techniques.** All *S. flexneri* mutant strains were derived by bacteriophage P1 transduction of the indicated *E. coli* mutations into the *S. flexneri* 2a strain 2457T. *E. coli* strains TRMG1655 (*csrA::Kan*), TWMG1655 (*csrC::Tet*), and RG1-B (*csrB::Cm*) were kindly provided by T. Romeo (16, 67). The *barA*, *uvrY*, *csrD* (*yhdA*), *cra*, and *pfkA* mutations were transduced from strains in the Keio collection (1). For each mutant, replacement of the wild-type allele was confirmed by PCR analysis.

**Recombinant DNA techniques.** Primers used in this study are listed in Table 2. The *csrA* expression plasmid pQCsrA was constructed by cloning the wild-type *S. flexneri* *csrA* gene under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter in the expression plasmid pQE2 (Qiagen, Valencia, CA). *S. flexneri* *csrA* was amplified from the chromosome of 2457T using primers *csrA*-fwd-BseRI and *csrA*-rev-HindIII. The amplified fragment was digested with BseRI and HindIII and cloned into similarly digested pQE2. To construct plasmids overexpressing *csrB* or *csrC*, the genes were amplified from 2457T using primers pQCsrB-fwd and pQCsrB-rev and pQCsrC-fwd and pQCsrC-rev, respectively, and initially cloned in pGEM-T Easy (Promega). The cloned fragments subsequently were excised using HindIII and EcoRI and ligated into similarly digested pQE2, thereby creating pQCsrB and pQCsrC.

pWPfkA was constructed by amplifying wild-type *pfkA* from *S. flexneri* 2457T using primers pWPfkA-fwd and pWPfkA-rev. The resulting PCR fragment was cloned into pGEM-T Easy, creating pGWPfkA. The *pfkA* fragment was excised from pGWPfkA using SalI and NotI and ligated into similarly digested pWSK29 (62). To construct pWCra, *cra* was amplified from the chromosome of 2457T using primers pWCra-fwd and pWCra-rev and then cloned into pGEM-T Easy. The *cra* fragment was excised from pGEM-T Easy by digesting with EcoRI and NotI and ligating the product into similarly cut pWKS30 (62). All plasmid inserts were sequenced using an ABI 3130 sequencer (Applied Biosystems) at the University of Texas at Austin DNA sequencing facility.

**Cell culture assays.** Invasion assays were performed using a modification of the method of Hong et al. (23). Briefly, CR<sup>+</sup> colonies from an overnight TSB agar plate containing 0.01% (wt/vol) Congo red were inoculated into LB broth and grown at 37°C with aeration to exponential phase (*A*<sub>650</sub>, 0.75 to 0.8). Approximately 2 × 10<sup>8</sup> bacteria were added to a subconfluent monolayer of Henle cells in 35-mm six-well polystyrene plates (Corning) and centrifuged for 10 min at 700 × *g*. The monolayers were incubated for 30 min at 37°C with 5% CO<sub>2</sub> and then washed with phosphate-buffered saline (PBS-D; 1.98 g KCl, 8 g NaCl, 0.02 g KH<sub>2</sub>PO<sub>4</sub>, and 1.4g K<sub>2</sub>HPO<sub>4</sub> per liter; pH 7.5) to remove extracellular bacteria. Two milliliters of MEM supplemented with 20 μg/ml gentamicin was added. Plates were incubated an additional 40 min, then washed with PBS-D and stained with Wright-Giemsa stain (Camco, Ft. Lauderdale, FL). Internalized bacteria were visualized by microscopy at ×600 magnification. At least 300 Henle cells were examined per well, and those containing three or more internal bacteria were scored positive for invasion.

To measure bacterial attachment, *S. flexneri* strains were grown as described for the invasion assay. Approximately 2 × 10<sup>8</sup> bacteria were added to a subcon-

fluent monolayer of Henle cells. Plates were centrifuged for 5 min at 700 × *g* and then incubated for 15 min at 37°C in 5% CO<sub>2</sub> prior to washing with PBS-D and staining as described above. Adherent bacteria were counted via microscopy. The total number of bacteria present per 100 Henle cells was recorded for each strain.

The plaque assays were performed as previously described (17, 22). In brief, 1 × 10<sup>5</sup> bacteria were added to confluent monolayers of Henle cells cultured in six-well polystyrene plates (Corning). Following the initial incubation, monolayers were washed and overlaid with MEM containing 0.3% glucose and 40 μg/ml gentamicin. After 48 h of incubation, wells were washed with PBS-D and stained with Wright-Giemsa.

**Analysis of Ipa.** *S. flexneri* strains were grown in LB broth to exponential phase as for the invasion assay, collected by centrifugation, and resuspended in Laemmli buffer at a concentration of 1 × 10<sup>10</sup> bacteria per ml. Samples were boiled for 5 min, and the proteins in lysates from equal numbers of cells were separated by SDS–12% polyacrylamide gel electrophoresis. Proteins were visualized by Coomassie brilliant blue staining (37) or transferred to a 0.45-μm-pore-size nitrocellulose membrane (Hybond-ECL; GE Healthcare) for Western blotting as described previously (18). Membranes were probed with mouse monoclonal anti-IpaB or mouse monoclonal anti-IpaC antibodies (generously provided by E. V. Oaks, WRAIR), followed by goat anti-mouse horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Signal was detected by developing the blot with a Pierce enhanced chemiluminescence (ECL) detection kit (Pierce).

**Analysis of lipopolysaccharide.** Lipopolysaccharide (LPS) extraction was performed following the protocol of Hitchcock and Brown (21). Strains were grown in LB broth as for the invasion assay, and 1 × 10<sup>9</sup> bacteria were harvested by centrifugation. Pellets were resuspended in 50 μl of lysis buffer (21) and boiled for 10 min. A total of 25 μg of proteinase K was added, and samples were incubated at 65°C for 1 h. Samples were boiled an additional 5 min and separated by 12% tricine-SDS-polyacrylamide gel electrophoresis. LPS was visualized by silver staining (58).

**Detergent sensitivity assay.** SDS sensitivity was determined as described by Hong et al. (22). Bacteria were grown in LB broth as for the invasion assay. The culture was centrifuged at 16,300 × *g* for 5 min and then resuspended in 25 mM Tris-Cl, pH 7.5, to a final *A*<sub>650</sub> of 1.0. SDS was added to a final concentration of 0.1% to one set of samples per strain. Samples were incubated at 37°C without aeration, and the ratio of absorbance at each time point (*T*<sub>n</sub>) versus the absorbance at time zero (*T*<sub>0</sub>) was determined and expressed as a percentage (relative to the *A*<sub>650</sub>). Data shown are from three independent experiments, each performed in triplicate.

**Real-time PCR.** RNA was isolated from exponential-phase bacteria grown as described for the invasion assay using the RNeasy mini kit (Qiagen) and then treated with DNase I (Invitrogen) to eliminate genomic DNA contamination. The RNA was quantified using an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE), and a total of 2 μg of RNA was used to make cDNA by using the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). cDNA was diluted 1:10 in diethyl pyrocarbonate-H<sub>2</sub>O, and 2.5 μl of diluted cDNA was used as template in a 25-μl real-time PCR mixture. TaqMan Universal master mix (Applied Biosystems) was used for all real-time PCRs. Primers and probes were designed using Primer Express (Applied Biosystems), and probes used were labeled with 6-carboxyfluorescein (6-FAM) and minor groove-binding nonfluorescent quencher (MGBNFQ). Reactions were performed in a 7300 real-time PCR system (Applied Biosystems) under standard reaction conditions. All data were normalized to the level of *dkcA* cDNA in the sample.

**Intracellular ATP.** The intracellular ATP levels of the wild-type and mutant strains of *S. flexneri* were measured using the ATP bioluminescence assay kit HS II (Roche, Mannheim, Germany) following the manufacturer's protocol. Strains were grown in LB broth to exponential phase as in the invasion assay, diluted 1:10 into dilution buffer, and lysed using the provided cell lysis reagent. Following addition of the luciferase reagent, the light signal was integrated for 10 s with a 1-s delay. All measurements were within the linear range as determined for an ATP standard curve.

## RESULTS

**An *S. flexneri* *csrA* mutant is defective in invasion.** To assess the role of CsrA in *S. flexneri* virulence, a *csrA* mutant was constructed by transduction of the *csrA::Kan* mutation from *E. coli* TRMG1655 (44) into *S. flexneri* 2457T. The transposon insertion site in TRMG1655 is located near the 3' end of the *csrA* open reading frame, and the strain has partial CsrA ac-

TABLE 3. CsrA and Cra influences on *S. flexneri* virulence in cell culture assays

Strain	Genotype	% wild type invasion <sup>a</sup>	Plaque morphology and relative no. <sup>b</sup>
2457T	Wild type	100	+
AGS120	<i>csrA::Kan</i>	5 ± 4	pp, <10% WT
AGS120/pQCsrA	<i>csrA::Kan/pQCsrA<sup>c</sup></i>	47 ± 6	pp, ~20% WT
AGS190	<i>cra::Kan</i>	131 ± 13	pp
AGS190/pWCra	<i>cra::Kan/pWCra</i>	90	+
AGS100	<i>barA::Kan</i>	98 ± 9	+
AGS110	<i>uvrY::Kan</i>	143 ± 10	+
AGS130	<i>csrB::Cm</i>	111 ± 10	+
AGS140	<i>csrC::Tet</i>	126 ± 5	+
AGS150	<i>csrD::Kan</i>	43 ± 10	<10% WT
2457T/pQCsrB	pQCsrB <sup>c</sup> (CsrB overexpression)	53	ND
2457T/pQCsrC	pQCsrC <sup>c</sup> (CsrC overexpression)	53	ND
AGS220	<i>pfkA::Kan</i>	0	-
AGS220/pWPfkA	<i>pfkA::Kan/pWPfkA</i>	77 ± 10	+

<sup>a</sup> Percentage of Henle cells with ≥3 intracellular bacteria. Invasion rates for wild-type (WT) strain 2457T ranged from 70 to 85% in different experiments, and values for the other strains were normalized to the invasion percentage of the wild type within each experiment. At least 300 Henle cells were counted in each experiment, and the values given are averages ± 1 standard deviation of three independent experiments each performed in triplicate. The data shown for complementation of *csrA* and *cra* and for *csrB* and *csrC* overexpression are averages of two independent experiments performed in triplicate.

<sup>b</sup> +, clear plaques of ~1.5 mm, number of plaques equivalent to WT (100 to 200 plaques/well); pp, pinpoint turbid plaques with diameter of <0.5 mm; -, no visible plaques; ND, not determined. The relative number of plaques (as a percentage) is based on the number of plaques formed by the WT in the same experiment.

<sup>c</sup> Expression was induced by addition of 200 μM IPTG.

tivity. TRMG1655 is defective in glycogen metabolism, leading to a 20-fold increase in glycogen stores (69) but, unlike a *csrA* null mutant, it shows wild-type growth in rich medium (44, 46, 55). The *S. flexneri csrA::Kan* mutant AGS120 had the same phenotypes as TRMG1655 in that it grew well in rich medium and appeared to have increased internal glycogen stores as detected by iodine staining (data not shown).

The *S. flexneri csrA* mutant was assessed for its effect on phenotypes correlated with *S. flexneri* virulence, including adherence, invasion, and cell-to-cell spread in cultured epithelial cells. The *csrA* mutant was severely impaired in invasion of Henle cells (Table 3). The invasion defect in the *csrA* mutant correlated with reduced attachment to the Henle cells, compared to the parental strain (Fig. 2A). As expected based on the attachment and invasion data, the *S. flexneri csrA* mutant formed fewer plaques in the epithelial cell monolayers (Table 3). Moreover, the plaques formed by the *csrA* mutant were smaller and more turbid than those of the wild type (Table 3), indicating a defect in intracellular multiplication or cell-to-cell spread.

Introduction of the cloned *csrA* gene on a plasmid only partially restored invasion and normal plaque phenotype (Table 3). The *csrA* mutant carrying the plasmid with the CsrA gene (pQCsrA) formed more plaques than the mutant strain, but these plaques were still smaller than those formed by wild-type *S. flexneri* (Table 3). Lack of full complementation may indicate that the level of CsrA produced by the plasmid is not optimal, or the chromosomal *csrA::Kan* allele may have a

dominant negative effect, since this mutation results in production of a protein with partial CsrA activity (55).

**Regulators of CsrA influence *S. flexneri* virulence.** To determine further the effects of CsrA levels on *S. flexneri* invasion and plaque formation, we constructed mutations in genes that alter the level of free CsrA. Because CsrB and CsrC sequester CsrA (Fig. 1A), we anticipated that loss of CsrB or CsrC would increase the amount of free CsrA. Disruption of *csrB* or *csrC* led to a modest increase in invasion by *S. flexneri* (Table 3), consistent with an increase in CsrA leading to more efficient invasion of the Henle cells. Conversely, overexpression of *csrB* or *csrC* from the IPTG-inducible promoter in pQE2 resulted in a decrease in invasion (Table 3), correlating with reduced CsrA levels. Another regulator of CsrA activity in *E. coli*, CsrD, promotes RNaseE-mediated degradation of CsrB and CsrC (Fig. 1A). Disruption of *csrD* decreases the decay rate of CsrB and CsrC, leading to increased levels of these RNAs and thus decreased levels of free CsrA (53). Consistent with *csrB* and *csrC* overexpression data, the *S. flexneri csrD* mutant was impaired in invasion compared to the wild type and formed fewer plaques than wild type (Table 3).

The BarA-UvrY two-component system stimulates expression of *csrB* and *csrC* (54, 67). In the absence of BarA or UvrY, levels of CsrB and CsrC decrease, thereby increasing the amount of free CsrA in *E. coli* (54, 67) (Fig. 1A). We therefore hypothesized that the absence of one or both components of the two-component system would increase Henle cell invasion by *S. flexneri*. A mutation in *uvrY* increased the percentage of Henle cells invaded by *S. flexneri*, consistent with decreased

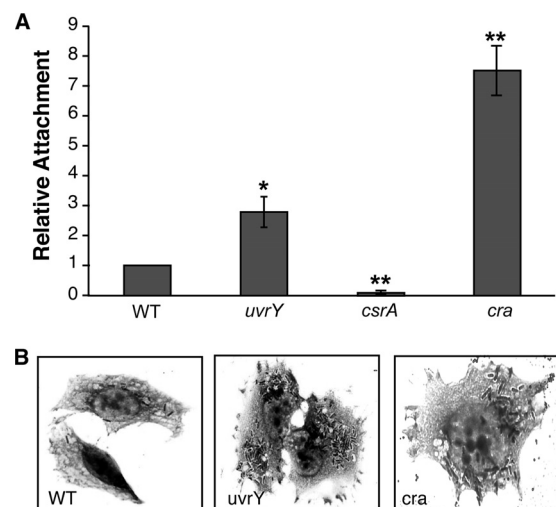


FIG. 2. CsrA and Cra influence *S. flexneri* attachment to epithelial cells. (A) Equivalent numbers of each strain were added to subconfluent monolayers and allowed to attach for 15 min prior to washing and staining. The total number of bacteria attached to 100 Henle cells was determined for each strain. Strains tested were 2457T (WT), AGS110 (*uvrY*), AGS120 (*csrA*), and AGS190 (*cra*). Values were normalized to the wild-type attachment level for each experiment. Wild-type adherence was 60 to 90 bacteria/100 cells (average, 0.8 bacteria/cell). Data presented are the mean values with standard deviations for three independent experiments. \*,  $P < 0.05$ , and \*\*,  $P < 0.01$ , compared to WT (by Student's *t* test). (B) Representative images from bacterial invasion of Henle cells by 2457T (WT), AGS110 (*uvrY*), or AGS190 (*cra*). Intracellular bacteria were visualized by Giemsa staining and microscopy.

expression of *csrB* and *csrC* and a resultant increase in CsrA activity (Table 3). Moreover, Henle cells invaded by the *uvrY* mutant contained greater numbers of intracellular bacteria compared to the wild type (Fig. 2B). Because of its increased invasion, we examined the attachment of the *uvrY* mutant to epithelial cells. The increased invasion was accompanied by an increased attachment of the *uvrY* mutant to Henle cells (Fig. 2A). The *barA* mutant did not demonstrate any difference in invasion compared to wild type (Table 1). However, UvrY may be phosphorylated by another histidine kinase in the absence of BarA, thus mitigating the effects of a *barA* mutation on *csrB* and *csrC* expression (64).

**Inhibition of *S. flexneri* invasion by the metabolic regulator Cra.** In contrast to CsrA, the transcriptional regulator Cra inhibits glycolysis and stimulates gluconeogenesis (Fig. 1A). We wanted to determine whether Cra would also have the opposite effect of CsrA on *S. flexneri* invasion. The *cra* mutant invaded a larger fraction of the Henle cells than the wild type (Table 3), and the invaded Henle cells each contained larger numbers of intracellular bacteria (Fig. 2B). Consistent with the increased invasion, the *cra* mutant showed a 7-fold increase in the average number of bacteria attached to each epithelial cell (Fig. 2A). In spite of the increased attachment and invasion, the plaques formed by the wild type were much smaller and more turbid than those made by the parental strain. In the invasion assay, it was noted that a large number of the intracellular bacteria appeared to remain within a vacuole (Fig. 2B), and this may contribute to inefficient plaque formation. Introduction of a plasmid carrying *cra* (pWCra) into the *cra* mutant reduced invasion to near-wild-type levels and restored wild-type plaque morphology (Table 3). The aberrant plaque morphology indicates that while Cra inhibits attachment and invasion, it is needed for optimal growth inside the host cell or for cell-to-cell spread.

**The glycolytic enzyme PfkI is necessary for *S. flexneri* invasion.** The phenotypes of the *csrA* and *cra* mutants indicate that efficient attachment correlates with the amount of free CsrA and positive regulation of glycolysis. To test directly whether the invasion phenotypes of these regulatory mutants were due to altered levels of glycolysis, we made a mutation in a gene required for glycolysis, *pfkA*, which encodes phosphofructokinase (PfkI) (Fig. 1B). *pfkA* expression is positively regulated by CsrA (46) and is negatively regulated by Cra (10). If the invasion phenotypes of the *csrA* and *cra* mutants are due to their effects on glycolysis, then the *pfkA* mutant should have the same phenotype as the *csrA* mutant. The phenotype of the *S. flexneri* *pfkA* mutant was consistent with this model; the mutant did not invade Henle cells and subsequently failed to form plaques (Table 3). Expression of *pfkA* from the plasmid pWPfkA restored invasion of the *pfkA* mutant to near-wild-type levels and also restored plaque formation (Table 1). The invasion and plaque phenotypes of the *pfkA* mutant suggest that glycolysis is needed for invasion and virulence.

**PfkI and CsrA affect the *S. flexneri* outer membrane.** A possible effect of altered carbon metabolism is changes in the structure of the *S. flexneri* LPS due to alterations in the intracellular pool of sugars available for LPS synthesis. Altered LPS affects virulence of *S. flexneri* (23, 36). IcsA, the bacterial protein needed for polar actin polymerization and intercellular spread, is aberrantly localized in rough mutants (36, 49, 59).

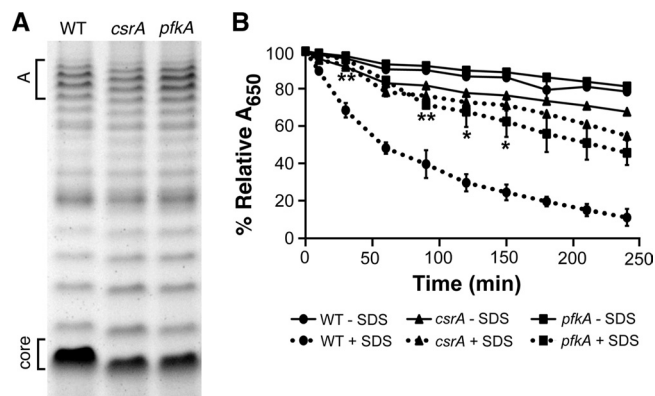


FIG. 3. Effects of CsrA and PfkA on *S. flexneri* LPS and sensitivity to SDS. (A) LPS was harvested from 2457T (WT), AGS120 (*csrA*), and AGS220 (*pfkA*). LPS samples from equal numbers of cells grown to exponential phase were separated on a 12% tricine-SDS-PAGE gel and visualized by silver staining. "Core" refers to the migration pattern of the LPS core without attached O-antigen repeats. Mode A is the LPS core with chains consisting of approximately 11 to 17 O-antigen repeats. (B) Strains 2457T (WT), AGS120 (*csrA*), and AGS220 (*pfkA*) were grown to exponential phase and washed with  $1 \times$  PBS-D. A final concentration of 0.1% SDS was added to samples at  $T_0$ . Absorbance ( $A_{650}$ ) was determined at 0 min, 10 min, and successive 30-min intervals for 4 h. The percent relative  $A_{650}$  was calculated as  $(A_{650} \text{ at } T_n)/(A_{650} \text{ at } T_0) \times 100$ . Data presented are the averages of three independent experiments performed in triplicate. Error bars represent standard deviations. Detergent sensitivity of the mutant strains was compared to wild type by using an unpaired Student's *t* test. \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ .

The length and degree of glycosylation of the LPS also influence invasion by altering exposure of the type III secretion system (TTSS) needle (23, 68). To determine whether there were differences in the LPS structure, the LPS profiles of the *csrA* and *pfkA* mutants were compared to the wild type (Fig. 3A). LPS molecules consisting of only the core migrate fastest in gel electrophoresis, while slower-migrating bands represent chains with increasing numbers of O-antigen repeats. Both the *csrA* and *pfkA* mutants produced LPS in which each repeat migrated faster than the wild type (Fig. 3A). The mutants had the typical pattern of chain length distribution, designated mode A (Fig. 3A), and both the *csrA* and *pfkA* mutants, like the wild type, had detectable amounts of the very long mode B chains (data not shown). This suggests that the LPS modifications seen in the *pfkA* and *csrA* mutants are a result of alterations in the lipid A or core oligosaccharide portion of LPS and not a change in the O-antigen distribution or length. A change in the cell surface of the *csrA* and *pfkA* mutants was also detected by measuring resistance to detergent. Both of these mutants were more resistant to SDS than the wild type (Fig. 3B). Deletion of *cra* had no discernible effects on the *S. flexneri* LPS profile or resistance to detergent (data not shown).

**Ipa synthesis and secretion by the *pfkA* and *csrA* mutants.** Changes in LPS structure may influence the interaction between *S. flexneri* and epithelial cells, but the complete loss of invasiveness of the *pfkA* mutant is similar to the phenotype of mutants defective in the synthesis or presentation of the virulence plasmid-encoded invasion proteins. These proteins, IpaA, -B, -C, and -D, and the TTSS proteins (Mxi and Spa), responsible for their secretion (7) are essential for *S. flexneri*

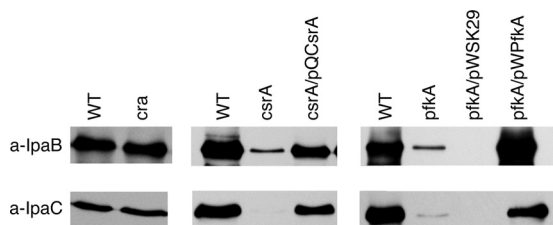


FIG. 4. Disruptions in glycolysis inhibit Ipa synthesis. (A) Strains tested were 2457T (WT), AGS120 (*csrA*), AGS220 (*pfkA*), AGS190 (*cra*), AGS120/pQCsrA, and AGS220, carrying either the empty vector (pWSK29) or the cloned *pfkA* gene (pWPfkA). Total cell extracts were prepared from exponential-phase cells and then analyzed by SDS-PAGE and immunoblotting with mouse monoclonal anti-IpaB or mouse monoclonal anti-IpaC antibody. Lysates from equal numbers of cells as determined by optical density were used.

attachment, invasion, and vacuole lysis (24). The levels of two of the invasion proteins, IpaB and IpaC, were examined in the *csrA* and *pfkA* mutants by immunoblotting with monoclonal antibodies against IpaB or IpaC. The *S. flexneri* *pfkA* and *csrA* mutants produced greatly reduced amounts of the effectors compared to the parental strain (Fig. 4). Complementation with the wild-type genes on plasmids restored synthesis of both invasion proteins (Fig. 4). The failure of the *csrA* and *pfkA* mutants to synthesize IpaB and IpaC is consistent with the impaired invasion of these strains (Table 3). In contrast, the *cra* mutant produced IpaB and IpaC at levels similar to wild type (Fig. 4), indicating that the hyperinvasive phenotype was not a result of oversecretion of the invasion proteins.

**Expression of *virB* and *virF* are decreased in the *csrA* and *pfkA* mutants.** Expression of the *ipa* genes is controlled by the VirF-VirB regulatory cascade, which modulates their expression in response to several environmental factors, including osmolarity, pH, and temperature (5, 24, 41, 57). VirF activates transcription of *virB*, and VirB, in turn, activates expression of the invasion loci, including the *ipa* operon. *virF* and *virB* expression levels were examined in the *csrA* and *pfkA* mutants. As determined by real-time PCR, the amounts of *virF* and *virB* mRNA were reduced 5- to 100-fold in the mutants. Complementation of the *pfkA* mutation with the wild-type gene on a plasmid restored expression to near-wild-type levels (Fig. 5). The loss of Ipa synthesis is consistent with the reduced expression of *virF*. Reduced levels of VirF would lead to reduced *virB* expression and subsequent loss of activation of the virulence operons.

The mechanism by which *csrA* and *pfkA* affect expression of *virF* and the downstream genes is not clear. *virF* expression is influenced by DNA supercoiling, and the ATP/ADP ratio affects supercoiling (61). To determine whether modifications in central carbon metabolism caused by mutation of *csrA* or *pfkA* affect energy production, the levels of intracellular ATP were measured in aliquots of the same cultures used for determining *vir* gene expression by real-time PCR. The *pfkA* mutant, and to a lesser extent the *cra* mutant, showed reductions in intracellular ATP levels, but the *csrA* mutant had ATP concentrations equal to or greater than the wild type (Fig. 6). Thus, the *pfkA* mutation resulted in lower ATP levels, but this cannot explain the loss of virulence gene expression, since the *csrA* mutant,

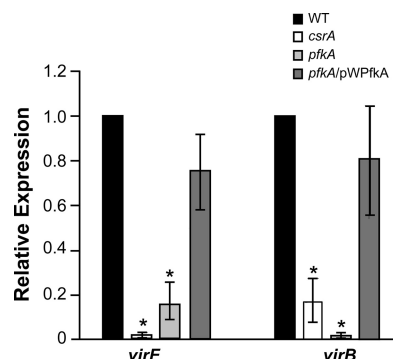


FIG. 5. Expression levels of the virulence regulators *virF* and *virB* are significantly reduced in the *csrA* and *pfkA* mutants. Real-time PCR analysis of *virF* and *virB* expression was performed using strains *S. flexneri* 2457T (WT), AGS120 (*csrA*), AGS220 (*pfkA*), and AGS220/pWPfkA (PfkI<sup>+</sup>). Values are normalized to *dksA* in each sample, and results shown are relative to the value obtained for 2457T. Data presented are the averages of three or more independent experiments, and error bars represent 1 standard deviation. Relative expression was compared to the wild type by using Student's *t* test. \*, *P* < 0.01.

which also reduced virulence gene expression, had normal ATP levels.

Alternatively, blocking glycolysis by mutation in *pfkA* may cause transient accumulation of the precursor, fructose-6-phosphate, or a decrease in the levels of intermediates or end products downstream of the block in the pathway. An increase in fructose-6-phosphate could lead to sugar phosphate stress, which is known to affect regulation of gene expression (13, 40, 60). To determine whether sugar phosphate stress affected expression of the *S. flexneri* virulence genes, the cells were grown in the presence of  $\alpha$ -methylglucoside (0.25, 0.5, or 1.0%) to induce sugar phosphate stress (28) and assayed for Congo red binding. No differences were seen in Congo red binding by the wild-type strain in media with or without  $\alpha$ -methylglucoside, indicating that accumulation of sugar phosphates did not suppress production of the virulence proteins.

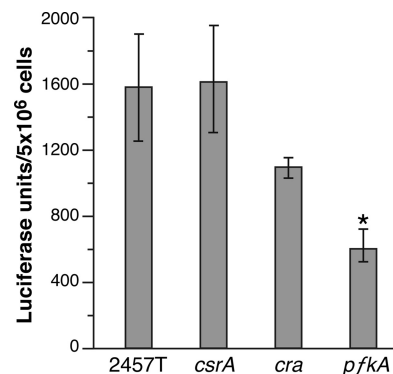


FIG. 6. Intracellular ATP levels in wild-type, *csrA*, and *pfkA* strains of *S. flexneri*. Samples of bacteria grown for measurement of virulence gene expression (Fig. 5) were lysed, and the intracellular ATP level was measured in a bioluminescence assay. ATP concentrations in the wild-type cells were  $\sim$ 1.2 mM, based on comparison to ATP standards. Data shown are the averages of three independent experiments. \*, *P* < 0.05 compared to the wild type (by Student's *t* test).

## DISCUSSION

*S. flexneri* encounters changes in environmental conditions during its transitions from the lumen of the gut to the subepithelial spaces and subsequently into the intracellular environment of the human host. To establish a successful infection, the bacterium must adapt to these environments, including coordinating expression of metabolic genes with nutrient availability. Acquiring and using available carbon sources is particularly important, and expression of genes involved in these pathways is highly regulated in the *E. coli/Shigella* group. We tested the role of the carbon regulators CsrA and Cra in *S. flexneri* virulence by constructing mutations in their genes and determining the phenotype of these mutants in cell culture assays. CsrA and Cra have opposite effects on glycolysis and gluconeogenesis, and mutations in the genes had opposite effects on the early stages of epithelial cell invasion. Mutation of *csrA*, which has been shown in *E. coli* to reduce glycolysis, led to decreased attachment and invasion, while the *cra* mutant, which had increased glycolysis, attached and invaded to a greater extent than wild type. This correlation between attachment/invasiveness and increased levels of CsrA could be seen throughout the CsrA regulatory circuit; mutations that led to increased CsrA (*csrB*, *csrC*, and *uvrY*) promoted attachment and invasion, while those that caused a decrease in CsrA (*csrA* and *csrD*) had reduced attachment and invasion to cultured cells. These data suggest that regulation of carbon metabolism and the metabolic state of the cell are critical determinants of *S. flexneri* invasion. A specific role of the glycolytic pathway was confirmed by making a mutation in *pfkA* which blocks glycolysis at an early step. The *pfkA* mutant showed reduced attachment and loss of invasion, indicating that a block in glycolysis led to reduced invasion.

Loss of PfkA led to several changes in *S. flexneri* that could result in reduced invasion (Fig. 7). The major effect was the reduced expression of the virulence gene regulators *virF* and *virB*. The *pfkA* mutant had a 5-fold reduction in *virF* expression, and *virB* expression, which is positively regulated by VirF, was reduced almost 100-fold. Similarly, the *csrA* mutant had significantly lower levels of *virF* and *virB* expression than the wild-type strain. In the absence of VirB, there was little synthesis of IpaB and IpaC, which are essential for *S. flexneri* invasion.

The mechanism by which loss of glycolysis affects *virF* expression is not yet known. The VirF-VirB regulatory cascade is regulated by several environmental cues, including pH, osmolarity, and temperature (5, 41, 56). These environmental inputs are thought to influence DNA supercoiling and in turn affect topology and accessibility of the *virF* promoter. Because DNA supercoiling also is affected by the cell's energy charge (61), we determined the ATP levels in the mutant and wild type. However, the normal ATP levels in the *csrA* mutant indicated that this is unlikely to be the cause of poor *virF* expression. The *csrA* and *pfkA* mutations result in changes in carbon flux through the metabolic pathways in the bacterial cell, and increased or decreased amounts of a metabolic intermediate or product of one of these pathways may be a regulatory signal for *virF* expression.

It is possible that the alterations in the LPS and outer membrane structure caused by the *csrA* or *pfkA* mutations might

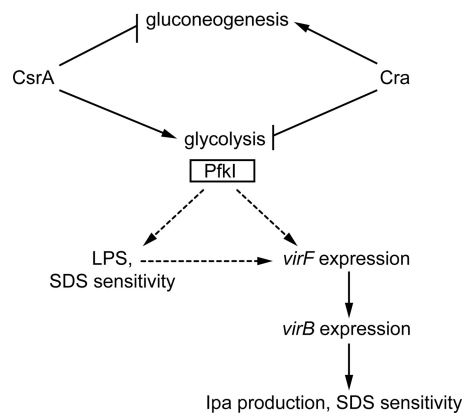


FIG. 7. Proposed model for regulation of *S. flexneri* pathogenesis by CsrA, Cra, and PfkI. Disruptions in glycolysis, caused by mutation of *csrA* or *pfkA*, reduce expression of *virB* and *virF*, leading to loss of Ipa production. Because all the virulence phenotypes of the *csrA* mutation are mimicked by the *pfkA* mutation, the effects of CsrA on attachment and invasion are likely due to its control of *pfkA*. Decreased expression levels of *ipa* and TTSS genes likely contribute to the increased resistance of the *csrA* and *pfkA* mutants to the detergent SDS. The altered LPS profile may also contribute to detergent resistance in the mutants, and these changes in the cell surface may influence the response of *S. flexneri* to environmental regulators of *virF* gene expression. Solid lines indicate known effects, and dashed lines indicate proposed effects based on the data presented.

affect the bacterium's ability to detect or respond to environmental signals such as osmolarity. Failure to detect the appropriate cues could prevent or delay the expression of *virF*. Alternatively, some changes in the outer membrane of the mutants, as measured by increased resistance to detergents, may be the result, rather than the cause, of reduced virulence gene expression. We had previously shown that there is a correlation between effector secretion and detergent sensitivity, as a Congo red-binding-negative strain defective in Ipa synthesis and secretion was more resistant to SDS than the virulent parent strain (22). The presence of the TTSS may reduce the stability of the outer membrane and increase sensitivity to detergents. Thus, the overall decrease in virulence gene expression in the *pfkA* and *csrA* mutants could account for both SDS resistance and decreased invasion. There was also a change in the migration of the LPS core. This change is not the direct result of decreased Ipa synthesis and secretion, since the Ipa synthesis mutant SA101 had the same LPS migration pattern as the wild type (data not shown). The altered LPS profile in the *csrA* and *pfkA* mutants may result from changes in the pools of sugars for LPS core modifications or altered expression of genes in the LPS biosynthetic pathway. These effects on LPS may further influence the interaction of *S. flexneri* with the host cells, as well as its ability to sense environmental cues for virulence gene expression.

In addition to the effect on invasion, the mutations reducing glycolysis also affected subsequent intracellular growth and cell-to-cell spread. The few plaques that were formed by the *csrA* mutant were smaller and more turbid than those of the wild type, suggesting a need for glycolysis subsequent to invasion. Reduced production of IpaB and IpaC may inhibit plaque formation in the *csrA* mutant, as these effectors have roles in both invasion and intercellular spread (33, 38). Synthesis of

IpaB and IpaC is downregulated during the early stage of intracellular growth (18), but their production is required later for intercellular spread of the bacteria (52).

Although the *cra* mutant was highly invasive, it produced small, turbid plaques, indicating a defect in intracellular growth or cell-to-cell spread. This effect may be the result of reduced gluconeogenesis or aberrant upregulation of glycolysis in the intracellular environment. Although the eukaryotic cytosol is seemingly nutrient rich, it is not sufficient to support growth of noninvasive *E. coli* cells when they are microinjected into the cytosol (14). Thus, *S. flexneri* must be specifically adapted to growth within the cytosol, and this likely involves the ability to use a variety of carbon sources for optimal growth in this environment.

There is increasing evidence supporting a role for carbon metabolism in virulence (12, 27, 31). Interruption of the glycolytic pathway or the phosphotransferase transport system inhibits replication of *Salmonella enterica* serovar Typhimurium within macrophage vacuoles (8). Carbohydrate metabolism has also been implicated in pathogenesis of enteroinvasive *E. coli* (EIEC), which is closely related to *Shigella*. In a cell culture model, an EIEC mutant defective in both glucose and mannose transport was significantly impaired in adherence and invasion (15). The intracellular pathogen *Listeria monocytogenes* appears to maximize its ability to grow intracellularly by using multiple carbon sources, such as glucose-6-phosphate and glycerol (26, 27). Glycolysis genes were downregulated and pentose phosphate cycle genes were upregulated in *Listeria* growing intracellularly, and mutational analysis supported a role for the pentose phosphate pathway during intracellular growth (27). Because of their roles in regulating these pathways, CsrA and Cra may exert an effect on invasion and replication of the bacteria within the host. Additionally, CsrA may regulate virulence gene expression more directly. In *Yersinia enterocolitica*, the Csr regulatory system regulates expression of the RovA virulence gene regulator, and a *csrA* mutant was defective in attachment to and invasion of HEp-2 cells (20). Similarly, *csrA* regulates the expression of virulence genes required for pedestal formation by enteropathogenic *E. coli* (6) and is required for expression of *Salmonella* pathogenicity island 1 (SPI-1) invasion genes (29). *S. flexneri* appears to have adopted similar strategies, using CsrA and Cra to control carbon metabolism and virulence gene expression during the course of infection, in order to initiate invasion and subsequently maintain its replicative niche inside the host epithelial cell.

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